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Inner Cell Mass-Specific Expression of a Cell Adhesion Molecule (PECAM-1/CD31) in the Mouse Blastocyst

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Platelet/Endothelial Cell Adhesion Molecule-1 (PECAM-1 or CD31) is thought to be a vascular-specific protein, but its function has not been clearly defined. Here, we demonstrate by using confocal immunofluorescence microscopy that PECAM-1 is first detected in the mouse blastocyst, which contains no vascular cells, and its expression is restricted to the pluripotent inner cell mass (ICM) cells. Expression is localized to cell–cell borders of the ICM and is detected at the very first signs of blastocoel formation. Consistent with these observations is that embryonic transcripts of PECAM-1 mRNA, as detected by RT-PCR, greatly increase during the morula-to-blastocyst transition and seven of the eight known alternatively spliced isoforms of PECAM-1 are expressed in the blastocyst. The synthesis of PECAM-1 is independent of compaction, cytokinesis, and DNA replication, as it is detected in embryos that are chronologically at the blastocyst stage following culture of 8-cell embryos in Ca^{2+}-free medium, or medium containing cytochalasin D or aphidicolin, respectively. By the late blastocyst stage, PECAM-1 expression is restricted to the pluripotent epiblast, at which point it has a mutually exclusive expression pattern to that of type IV collagen, a basement membrane marker. The reduction in PECAM-1 transcripts in retinoic acid-induced differentiation of F9 teratocarcinoma cells, a model of epiblast-to-primitive endoderm differentiation, confirmed the epiblast-specific expression of PECAM-1. By the egg cylinder stage of development, at which point the epiblast is no longer pluripotent, PECAM-1 is not detected. This ICM-specific pattern of expression suggests a novel developmental role of PECAM-1 that is independent of its function in vascular ontogeny.

Key Words: **blastocyst; ICM; PECAM-1; CD31; cell adhesion; preimplantation development; pluripotent.**

INTRODUCTION

One of the first cellular differentiation events in the mouse embryo occurs during the last day of preimplantation development as the morula transforms into the blastocyst approximately 3.5 days post coitum (dpc). The outer cells of the 32-cell morula become a fluid-transporting epithelium, the trophectoderm (TE), that is responsible for the formation of the blastocoel, the fluid-filled cavity of the blastocyst (reviewed in Fleming et al., 1998). The inner cells of the morula become the inner cell mass (ICM) cells of the blastocyst. These pluripotent cells give rise to all tissues of the embryo proper and to extraembryonic tissues. They are not totipotent, however, like their precursor cells of the morula that can contribute to the TE and its placental derivatives (Gardner and Nichols, 1991). This suggests that a developmental signal has occurred that differentiates the totipotent cells of the morula from the pluripotent cells of the ICM. The initiation of ICM-localized transcription of the genes FGF4, UTF1, and osteopontin may reflect such a signal (Botquin *et al.*, 1998; Niswander and Martin, 1992; Okuda *et al.*, 1998).

By 4.5 dpc, the blastocyst begins to implant in the uterine wall. At this point, the outer cells of the ICM that are in contact with the blastocoel differentiate into the primitive endoderm; these cells contribute exclusively to extraembryonic lineages. The remaining ICM cells are classified as the epiblast or primitive ectoderm, and remain pluripotent, giving rise to the embryo proper and the germ cells, as well

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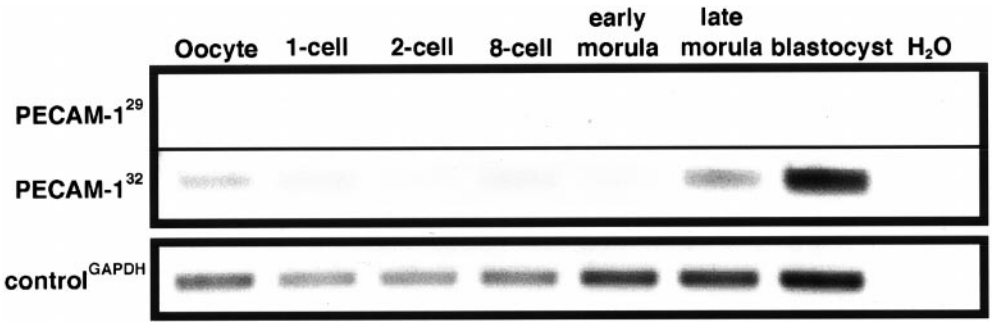


FIG. 1. Developmental profile by RT-PCR of PECAM-1 mRNA expression in the preimplantation embryo. Relative levels of PECAM-1 cDNA from the oocyte through to the blastocyst are shown after 29 (top) and 32 (middle) rounds of amplification. This is compared to a GAPDH control amplified for 30 cycles (bottom). Volumes representing embryo equivalent amounts of the amplification reactions are displayed using the inverted, ethidium bromide-stained images of the gels.

as contributing to components of the extraembryonic tissues. From 4.5 to 5.5 dpc, the epiblast transforms from a solid ball of rounded cells into cuboidal/columnar cells that form the egg cylinder (see Kaufman and Bard, 1999 for summary of cell fates). A molecular switch in the cells of the epiblast appears to have occurred during this transition from the late blastocyst to the early egg cylinder as these cells lose their ability to colonize the blastocyst and to form embryonic stem (ES) cells (Gardner and Brook, 1997).

Despite the importance of the ICM/epiblast in early mouse development and the potential application of its *in vitro* counterpart, the ES cell, little is known about the molecular characteristics of this cell population. The transcription factors Oct-4 and Sox-2 play a role in coordinating the ICM-specific expression of *UTF1*, *FGF4*, and osteopontin (Botquin *et al.*, 1998; Nishimoto *et al.*, 1999; Yuan *et al.*, 1995), but both of these transcription factors are also expressed prior to blastocyst formation (Schöler *et al.*, 1989; Robin Lovell-Badge, personal communication). Thus, their expression alone is not sufficient for ICM-specific gene transcription and further work is clearly required to understand ICM-restricted gene expression patterns.

Molecules involved in cell–cell and cell–matrix interactions are presumably important in defining and maintaining the properties of the ICM and directing its future differentiation (Damsky *et al.*, 1993). The early embryonic lethality due to the arrest of ICM differentiation in the β1 integrin and laminin γ1-null mice emphasizes the importance of such interactions at these formative stages (Smyth *et al.*, 1999; Stephens *et al.*, 1995). Nevertheless, no ICM-specific cell adhesion or membrane receptor molecules have been identified to date.

Platelet/endothelial cell adhesion molecule-1 (PECAM-1)—also known as CD31—is a plasma membrane-spanning molecule that contains six Ig-like extracellular domains, a short transmembrane region, and a complex cytoplasmic domain (Albelda *et al.*, 1990; Newman *et al.*, 1990). The extracellular domains are capable of forming both homophilic and heterophilic interactions with molecules on neighboring cells (DeLisser *et al.*, 1994). The cytoplasmic domain is derived from seven exons, and alternative splicing of three of these can give rise to eight isoforms of PECAM-1 (Baldwin *et al.*, 1994; Sheibani *et al.*, 1999). In

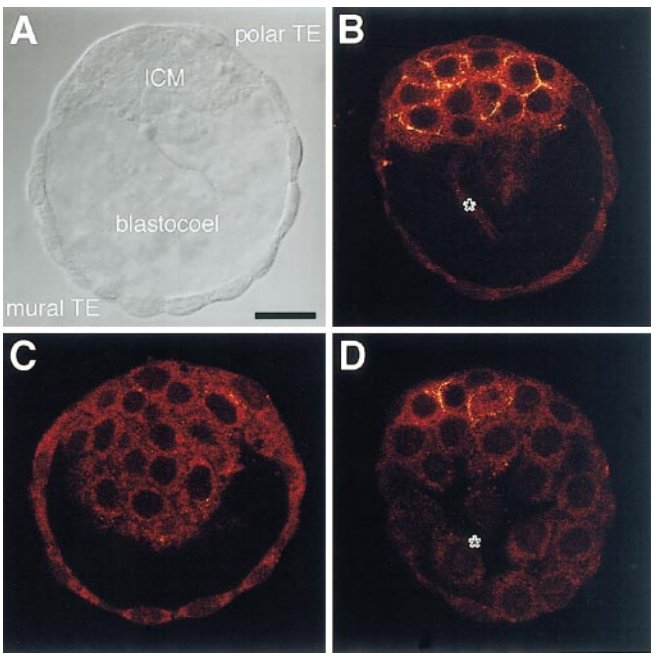


FIG. 2. Immunofluorescence detection of PECAM-1 in the blastocyst. A DIC image shows the morphology of a blastocyst (A) with the location of the polar trophectoderm (polar TE), the mural trophectoderm (mural TE), the inner cell mass (ICM), and the blastocoel cavity indicated. Confocal images of blastocysts immunostained with Mec13.3, a monoclonal antibody to mouse PECAM-1 (B and D), and with the 2° antibody alone (C) as a negative control. (B, D) Images of the same blastocyst but at different planes on the Z-axis: (B) through the center; (D) nearer the surface of the embryo and hence the blastocoel cavity is not clearly visible. The structure indicated by the asterisk (*) is a result of an in-folding of the mural TE into the blastocoel during the mounting process. All images are of equal magnification and the scale bar represents 25 μm.

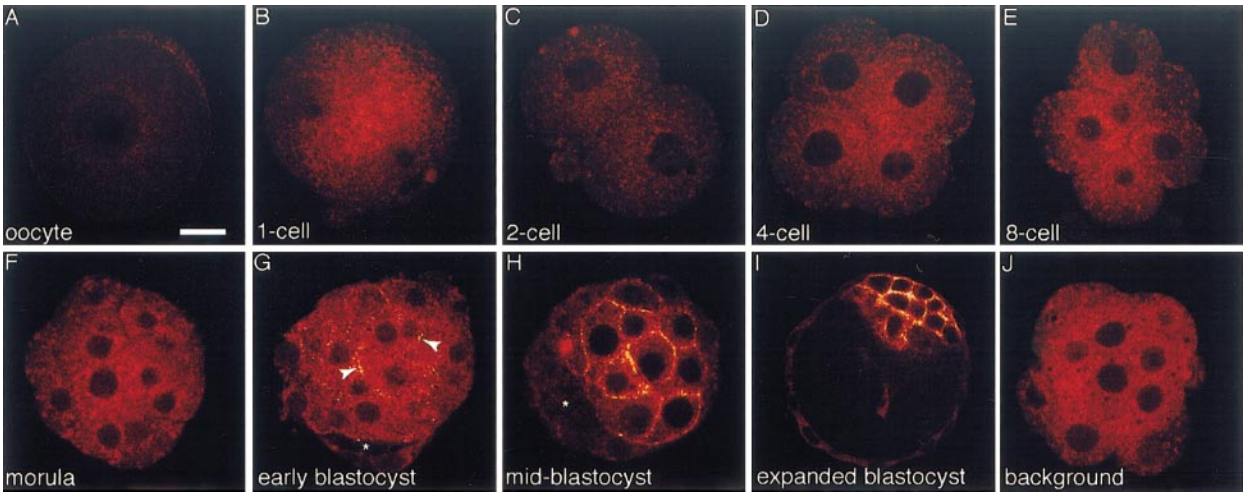


FIG. 3. Developmental profile of PECAM-1 protein expression in the preimplantation embryo. Confocal images of PECAM-1 immunostained embryos (Mec13.3 1° antibody) at progressive stages of mouse preimplantation development (A–I). For a background control, a morula is shown stained with 2° antibody alone (J). PECAM-1 staining at cell–cell borders becomes evident at the initial stages of blastocoel (*) formation (G, indicated by arrowheads). ICM-specific expression rapidly increases as the blastocyst expands (H, I). All images are of equal magnification and the scale bar represents 25 μm.

the postgastrulation embryo, expression of PECAM-1 is primarily restricted to cells of the vasculature, e.g., endothelial cells, monocytes, neutrophils, platelets, and particular T-cell subsets. The first appearance of PECAM-1 in the postgastrulation mouse embryo is on newly forming endothelial cells in the embryonic yolk sac (embryonic day 7.5) and later in the embryo proper (Baldwin *et al.*, 1994; Vecchi *et al.*, 1994).

PECAM-1 is implicated in several vascular-specific functions (for a review of the biology of PECAM-1 see DeLisser *et al.*, 1997a), and yet a precise role for PECAM-1 in any of these functions remains elusive. While initially characterized as a cell adhesion molecule, recent reports also suggest a role in signal transduction as the cytoplasmic domain can bind to a number of signaling molecules, including SHP-2, β-catenin, and γ-catenin (Ilan *et al.*, 2000; Jackson *et al.*, 1997; Pumphrey *et al.*, 1999). Thus, there is substantial evidence that PECAM-1 plays a role in both cell adhesion and signal transduction within the vascular compartment.

We report here the temporal and spatial pattern of PECAM-1 expression in the preimplantation mouse embryo. The protein is first detected in the blastocyst and its expression is restricted to the ICM cells. At 4.5 dpc, its expression is limited to the epiblast and, by 5.5 dpc, is no longer detected in the embryo. PECAM-1 expression is independent of compaction, cytokinesis, and DNA replication. Furthermore, PECAM-1 mRNA is detected in the morula, i.e., just prior to detection of PECAM-1 protein, and seven of the eight alternatively spliced isoforms of PECAM-1 mRNA are present in the blastocyst. These results define PECAM-1 as the first cell adhesion molecule to be ICM-specific and suggest a potential role for this molecule prior to vascular differentiation.

MATERIALS AND METHODS

Embryo Collection and Culture

All embryos were derived from superovulated CF-1 mice (Harlan) mated to B6D2F1/J males (Jackson Laboratory). Embryos were collected in bicarbonate-free MEM media (in which bicarbonate was replaced with 25 mM Hepes, pH 7.4; Life Technologies) containing 3 mg/ml polyvinylpyrrolidone (PVP). Embryos were cultured in 50-μl drops of KSOM containing 0.5× essential and nonessential amino acids (Life Technologies) under paraffin oil in an atmosphere of 5% CO_2 in air at 37°C as previously described (Ho *et al.*, 1995). Calcium-free KSOM containing amino acids was prepared by omitting the $CaCl_2$. To inhibit cytokinesis or DNA synthesis, embryos were cultured in KSOM containing 0.5 μg/ml cytochalasin D (Sigma) or 2.5 μg/ml aphidicolin (Sigma), respectively. For all experiments, at least 10 embryos were used per data point.

Cell Culture

F9 teratocarcinoma cells (ATCC) were cultured in DMEM media with 10% FBS and antibiotic on 90-mm tissue culture dishes coated with 0.1% gelatin. For immunofluorescence, cells were grown on gelatin-coated glass coverslips prior to fixation. Total RNA was isolated from F9 cells cultured for 0, 25.5, 49, 72.5, and 96 h in 1 μM retinoic acid using an RNeasy Mini kit (Qiagen).

Immunocytochemistry and Confocal Microscopy

The zona pellucida was first removed by a brief treatment with acid Tyrode's solution. Embryos were briefly washed in phosphate-buffered saline containing 3 mg/ml PVP (PBS/PVP) and fixed in 2.5% paraformaldehyde (PFA) in PBS for 15 min at room temperature. Fixed embryos were stored at 4°C in PBS/PVP. All subsequent steps involved washing the embryos through microdrops held at room temperature in a humidified chamber. Initially, embryos were placed in blocking solution (0.1% BSA, 0.01% Tween 20, PBS) for 15 min; this solution was used in all subsequent steps. The embryos were incubated for 60 min in 1.5 μg/ml Mec 13.3 (Pharmingen), which is a monoclonal antibody that recognizes an extracellular domain of PECAM-1, washed three times (15 min for each wash), and incubated for 1 h with the appropriately conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories), followed by a final three washes, each for 15 min. Type IV collagen staining followed a similar procedure but using a 1/500 dilution of a rabbit anti-mouse polyclonal antibody (Chemicon).

Vectashield mounting solution (Vector Laboratories) was used to mount the embryos onto slides. Nuclear staining was accomplished by adding Vectashield with DAPI to total 1/10 the mounting solution volume. To maintain blastocyst structure, the embryos were passed through increasing concentrations of mounting solution (25, 50, and 75%, diluted with PBS/PVP) for 15–30 min. Following transfer to 100% mounting solution, the embryos were then placed on the slide in ~5 μl. Coverslips were placed over the embryos onto four spots of Vaseline and sealed with nail polish. Fluorescence was detected on a Leica SP laser-scanning confocal microscope.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from 95 oocytes, 56 one-cell, 200 two-cell, 107 uncompacted eight-cell, 160 early morula (76 h post hCG), 66 late morula (85 h post hCG), and 143 expanded blastocysts by using Qiagen's RNeasy Mini Kit. Sigma's enhanced Avian RT-PCR kit was used for reverse transcription primed with oligo(dT). In each amplification reaction, cDNA representative of two embryo equivalents was used and equivalent amounts of the reactions were compared by gel electrophoresis. At these early stages of development, embryo equivalents are the most suitable and preferred method of comparison since there is no growth between the one-cell and blastocyst stages. Nevertheless, the relative amounts of GAPDH mRNA were also included to insure consistency in RNA isolation and in the reverse transcription reaction. Also, for consistency, RNAs from all the developmental time points sampled were purified at the same time from frozen, pooled embryos.

The standard primer set for PECAM-1 amplification consisted of a 5′ primer to exon 4 (5′-AGGGGACCAGCTGCACATTAGG-3′) and a 3′ primer to exon 6 (5′-AGGCCGCTTCTCTTGACCACTT-3′); when annealed at 58°C, these primers result in a single 452-bp amplification product. To delineate the alternatively spliced cytoplasmic domain isoforms of PECAM-1, primers were designed to span exon–exon junctions in the region of alternative splicing. The sequences of these primers were as follows with numbers in parentheses referring to the corresponding exon junction and U and L referring to 5′ and 3′ primers, respectively: ACAGCCATTACGGTTATG (11/12U), AACAGCCATTACGACCC (11/13U), CTTCCGTTCTTTGGTGA (13/16L), CTTCCGTTCTAGGGTCG (14/16L), and CCTTCCGTTCTAGAGTATC (15/16L). The primer sets (ps) and corresponding annealing temperatures were as follows: ps1 = 11/12U and 15/16L at 62°C; ps2 = 11/13U and 15/16L at 62°C; ps3 = 11/12U and 14/16L at 60°C; ps4 = 11/13U and 14/16L at 58°C; ps5 = 11/12U and 13/16L at 60°C; ps6 = 11/13U and 13/16L at 58°C. Specificity of these primer sets was confirmed by testing each on all eight cDNA isoforms. Seven of the eight isoforms' cDNAs had previously been cloned (Baldwin *et al.*, 1994) and the eighth (Δ12,14) was a gift from Nader Sheibani (Washington University, St. Louis, MO). A total of 3.2 embryo equivalents of blastocyst cDNA were used as template for each set of primers and amplification was for 40 cycles. The appropriate cDNAs were used as positive controls and water as a negative control. The identity of the Δ12, Δ14, Δ15, Δ12/14, Δ14/15, and the full-length amplification products from the blastocyst were confirmed by sequencing.

GAPDH 5′ (GCATGGCCTTCCGTGTTCCT) and 3′ (CCCTGTTGCTGTAGCCGTATTCAT) primers yielded a single product of 282 bp at 58°C; Oct-4 5′ (ACCGCCCCAATGCCGTGAAGTT) and 3′ (TGGGGGCAGAGGAAAGGATACAGC) primers yielded a single product of 593 bp at 62°C; Sox-2 5′ (GCTCACGGCGCGGCACAGATG) and 3′ (CCAGTTCGCAGTCCAGCCCTCACA) primers yielded a single product of 412 bp at 62°C; tPA 5′ (TTGGGCAGAACATACAGGGTGGTC) and 3′ (CTGGCATGCATCGTGGAGGTCTT) primers yielded a single product of 414 bp at 62°C. The identity of the Oct-4, Sox-2, and PECAM-1 amplicons from the undifferentiated F9 cells and the tPA and GAPDH amplicons from the differentiated F9s after 4 days of RA treatment were all confirmed by sequencing. All amplification reactions were performed on a Gradient Robocycler (Stratagene) by using *Taq* DNA polymerase (Life Technologies). All reactions used an extension temperature of 72°C for 1 min, a denaturation temperature of 94°C for 45 s, and the above-mentioned annealing temperatures for 45 s. The number of cycles for each reaction is given in Results.

RESULTS

Temporal Pattern of PECAM-1 mRNA Expression in Preimplantation Development

PECAM-1 transcripts had previously been identified in ES cells (Vittet *et al.*, 1996). Since ES cells are derived from and maintain the same pluripotency as the ICM (Bradley *et al.*, 1984; Evans and Kaufman, 1981; Martin, 1981), we anticipated that PECAM-1 mRNA would also be found in the blastocyst. A primer set that spanned exon boundaries was designed for use in RT-PCR analysis to amplify a single product corresponding to the extracellular domain of PECAM-1. Amplification of blastocyst cDNA using this primer set produced a single amplicon of the appropriate size (see blastocyst lane in Fig. 1). The sequencing results from this amplicon confirmed our prediction that PECAM-1 mRNA was present in the blastocyst.

We extended this RT-PCR analysis to earlier stages of development to ascertain whether PECAM-1 was expressed prior to blastocyst formation. Total RNA isolated from oocytes and preimplantation embryos at different stages of development were analyzed. The results from this analysis indicated that PECAM-1 transcript was most abundant in the blastocyst. After 29 cycles of amplification, PECAM-1 transcript was only observed in the blastocyst (Fig. 1). With

further amplification (32 cycles), PECAM-1 expression could be detected in the late morula. The increase in mRNA from the morula to the blastocyst may in fact be more pronounced than represented here since the early blastocoel is undetectable under the dissecting microscope and embryos undergoing initial blastocoel formation may have been included with the late morula stage embryos during embryo collection. This did occur for similar embryo collections used for immunostaining (see below).

Maternally derived transcripts, which were apparent in the oocyte after 32 cycles of amplification, were essentially degraded by the two-cell stage. The destruction of maternal PECAM-1 transcripts is similar to the widespread destruction of most maternal mRNAs that occurs between the oocyte and two-cell stage (Schultz, 1993 and references therein). There appeared to be a slight increase at the eight-cell stage, but the largest increase in PECAM transcript was seen from the late morula-to-blastocyst transition. This PCR was repeated a total of four times with similar results, therefore these findings are not the result of PCR artifacts.

PECAM-1 Protein Expression Is Restricted to the ICM

There are three anatomically identifiable cell types in the blastocyst: the mural trophectoderm (TE not in contact with the ICM), the polar trophectoderm (TE in contact with the ICM), and the ICM (Fig. 2A). We therefore employed laser-scanning confocal microscopy using a monoclonal antibody to an extracellular domain of murine PECAM-1 (Mec13.3) to localize PECAM-1 expression in the blastocyst. PECAM-1 expression was detected in the ICM, and staining was specific to cell–cell borders (Fig. 2B). In striking contrast, the mural TE was negative for PECAM-1 (Fig. 2D). The polar TE also appeared to be negative for PECAM-1, at least at cell surfaces other than TE cell–ICM cell junctions. It could not be established whether the staining at polar TE/ICM borders was solely due to ICM-specific expression.

When initially optimizing the immunofluorescence procedure, we also used another PECAM-1 antibody, mAb 390 (Baldwin *et al.*, 1994). We observed a similar signal to that of Mec13.3 when shorter fixation times (1–7 min) were used (data not shown). These results provided an independent confirmation of PECAM-1 expression in the blastocyst. Since the epitope for this antibody was lost upon longer fixation times, the Mec13.3 antibody was used in all subsequent experiments.

Temporal Pattern of PECAM-1 Protein Expression during Preimplantation Development

The observations that PECAM-1 was restricted to the ICM and its transcripts substantially increased from the morula to blastocyst transition led us to investigate PECAM-1 protein localization throughout preimplantation development. Accordingly, we analyzed PECAM-1 expression by laser-scanning confocal immunohistochemistry on oocytes and embryos at different developmental stages. In these experiments, 10–20 oocytes/embryos were analyzed at each developmental stage.

PECAM-1 protein staining in oocytes, one-cell, two-cell, four-cell, eight-cell, and morula (16- to 32-cell) embryos (Figs. 3A–3F) all had levels of fluorescence similar to that of the negative controls (i.e., embryos at the same stage of development but stained with the 2° antibody alone; see Fig. 3J as an example). There was no detectable cell–cell border staining of PECAM-1 at these developmental stages. Strikingly, PECAM-1 staining at cell–cell borders of the ICM appeared at the very initial stages of blastocoel formation, i.e., as soon as this cavity became detectable by confocal microscopy (Fig. 3G). Indeed, a number of embryos at this stage were initially classified as late morula, as the blastocoel was not apparent under the dissecting microscope during embryo collection; the cavity only became visible when the embryos were examined by confocal microscopy. The fluorescence signal from PECAM-1 rapidly increased as the blastocoel expanded in mid (Fig. 3H) and fully expanded blastocysts (Fig. 3I).

Expression of PECAM-1 Is Not Directly Linked to Compaction, Cytokinesis, or DNA Synthesis

The mechanism underlying the timing of blastocoel formation in the mouse embryo still remains unclear. There appears to be an intrinsic molecular clock controlling blastocoel formation that is independent of the absolute cell number, the number of cytokineses, the number of DNA replicative cycles, and the nucleo-cytoplasmic ratio (Dean and Rossant, 1984; Smith and McLaren, 1977; Surani *et al.*, 1980; Tarkowski and Wroblewska, 1967). To determine whether PECAM-1 localization to the cell–cell borders of the ICM was also linked to this intrinsic molecular clock, we attempted to affect its expression by independently altering compaction, cytokinesis, and DNA synthesis.

Culturing uncompacted eight-cell embryos in Ca^{2+}-free media disrupts compaction, which is a Ca^{2+}-dependent, E-cadherin-mediated event (Vestweber and Kemler, 1985) that occurs at the eight-cell stage. Embryos treated as such continue to divide but do not compact or form a blastocoel. In these experiments, when control embryos (i.e., eight-cell embryos cultured in Ca^{2+}-containing media) had formed expanded blastocysts, all embryos in both groups were analyzed for PECAM-1 expression. The embryos cultured in Ca^{2+}-free media, although not forming any blastocoel, did have localized PECAM-1 staining at cell–cell borders (Fig. 4B), although not all cells were PECAM-1-positive.

To ascertain whether PECAM-1 expression was linked to cell division, early morula (16-cell) were cultured in cytochalasin D. This drug inhibits cytokinesis and blastocoel formation, but not karyokinesis; the temporal sequence of

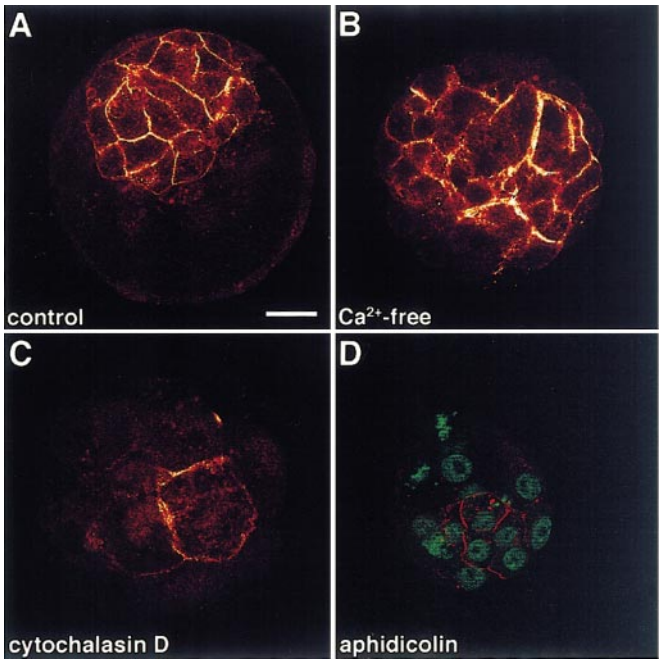


FIG. 4. PECAM-1 expression is not dependent on compaction, cytokinesis, or DNA synthesis. Confocal images of PECAM-1-immunostained embryos. (A) Control blastocyst derived from cultured eight-cell embryo. (B) Embryo cultured in Ca^{2+}-free media from the uncompacted eight-cell stage. (C) Embryo cultured in media containing 0.5 μg/ml cytochalasin D from the uncompacted eight-cell stage. (D) Shortly after compaction, embryos were cultured for 9 h in medium containing 2.5 μg/ml of aphidicolin. The embryos were then transferred to aphidicolin-free media and cultured until blastocyst formation. These blastocysts, which were also stained with DAPI (green nuclei), clearly contain fewer cells then the control embryos. All images are of equal magnification and the scale bar represents 25 μm.

the treated embryo's molecular development remains the same as that of untreated cells (Pratt *et al.*, 1981). All embryos were analyzed for PECAM-1 localization after control embryos (i.e., early morula cultured without cytochalasin D) formed fully expanded blastocysts. PECAM-1 staining, although relatively less than control embryos, was evident on some but not all of the large multinucleated cells of the cytochalasin D-treated embryos (Fig. 4C).

To block DNA synthesis, early morula (16-cell) were cultured in aphidicolin. Embryos treated as such form blastocysts with fewer cells (Dean and Rossant, 1984). Since prolonged treatment with aphidicolin results in embryo death (Dean and Rossant, 1984), the embryos were cultured in the presence of aphidicolin for 9 h, which is long enough to prevent at least one cycle of DNA replication, prior to transfer to aphidicolin-free medium. The embryos were then cultured and analyzed for PECAM-1 protein expression at a time when control embryos (i.e., early morula cultured without aphidicolin) formed fully expanded blastocysts. As expected, the aphidicolin-treated embryos formed blastocysts with fewer cells (~23 cells per blastocyst) compared to the control embryos (~35 cells per blastocyst). Nevertheless, PECAM-1 in aphidicolin-treated embryos localized to cell–cell borders specific to the inner cell mass (Fig. 4D).

Alternatively Spliced Isoforms of PECAM-1 Present in the Blastocyst

The cytoplasmic, C-terminal end of the PECAM-1 molecule is 120 amino acids long and spans 8 exons, 3 of which are alternatively spliced (exons 12, 14, and 15) in the postgastrulation mouse embryo (Baldwin *et al.*, 1994; Sheibani *et al.*, 1999). To determine the alternative splicing combinations that were present in the ICM of the blastocyst, we designed six primer sets that could distinguish all eight isoforms (see Materials and Methods for details) and used these in RT-PCR analysis of blastocyst total RNA. Seven of the eight known spliced isoforms were observed, only the Δ12,15 isoform was not detected (Fig. 5). Although this method was not directly designed for quantitation, comparison of isoform amplification from the blastocyst to that from plasmid templates suggest that Δ14,15 and Δ15 are the most abundant isoforms, which is consistent with that observed in endothelial cells (Sheibani *et al.*, 1999).

Peri- and Early Postimplantation Expression of PECAM-1

As PECAM-1 expression was restricted to the undifferentiated cells (ICM) of the 3.5 dpc blastocyst and was not detected in the differentiated cells of the TE, we were interested in seeing whether PECAM-1 expression remained restricted to the undifferentiated cell population upon further development of the embryo. By 4.5 dpc, the primitive endoderm, the cell layer in contact with the blastocoel, has differentiated from the ICM. The remainder of cells from the ICM (i.e., the epiblast/primitive ectoderm) stay undifferentiated/pluripotent. Therefore, we analyzed PECAM-1 protein expression in blastocysts that had been rigorously flushed from the uterine wall at embryonic day 4.5. In these embryos, PECAM-1 expression appeared to be restricted to the epiblast (Fig. 6A), since a single layer of PECAM-negative cells (the primitive endoderm) was present between the cavity and the PECAM-positive cells appropriately located to be defined as the epiblast.

To confirm that PECAM-1 was localized to the epiblast, we tried dual-labeling experiments using the PECAM-1 antibody in combination with antibodies to other blastocyst cell-type-specific antigens. Dual labeling with SSEA-1 or Troma-1 was unsuccessful. However, a basement membrane is located between the mural TE and the blastocoel (Thorsteinsdóttir, 1992), therefore we pursued dual immunostaining with type IV collagen, a basement membrane collagen. Confocal images from embryos stained only for Type IV collagen revealed an extensive basement mem-

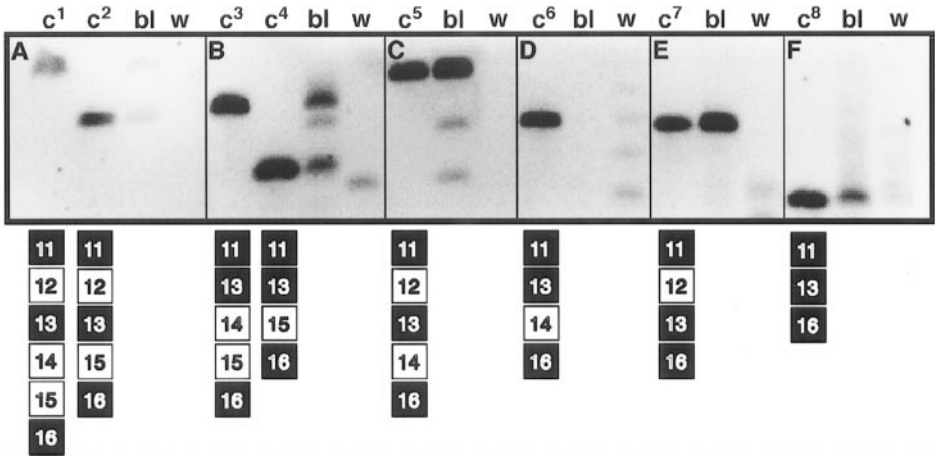


FIG. 5. PECAM-1 alternatively spliced isoforms present in the blastocyst. Six different primer sets (ps) designed to distinguish the eight different isoforms of PECAM-1 were used in RT-PCR analysis of blastocyst total RNA (bl). These were compared to positive control cDNA ($c^{\#}$) and negative control water (w). The inverted, ethidium bromide-stained image of the amplification products is shown here. (A) ps1 recognizes the wt (c^1) and Δ14 (c^2) isoforms, both of which are present in the blastocyst. (B) ps2 recognizes the Δ12 (c^3) and Δ12,14 (c^4) isoforms, both of which are present in the blastocyst. (C) ps3 recognizes the Δ15 isoform (c^5), which is present in the blastocyst. (D) ps4 recognizes the Δ12,15 isoform (c^6), which is not present in the blastocyst. (E) ps5 recognizes the Δ14,15 isoform (c^7), which is present in the blastocyst. (F) ps6 recognizes the Δ12,14,15 isoform (c^8), which is present in the blastocyst. A schematic of the clones is shown below with the alternatively spliced exons in white.

brane with a circular patch of cells distinctly devoid of type IV collagen in the location of the epiblast (Fig. 6B). From another harvest of 4.5 dpc embryos, dual-staining experiments for PECAM-1 and type IV collagen clearly indicated a mutually exclusive expression pattern between these two proteins (Figs. 6D–6F). From these same embryos, nuclear staining by DAPI suggested a PECAM-1-negative cell layer between the epiblast and the blastocoel, a location consistent with the primitive endoderm (Fig. 6C).

To confirm that PECAM-1 was restricted to the epiblast and not found in the primitive endoderm, we utilized the retinoic acid-induced differentiation of ICM/epiblast-like F9 teratocarcinoma cells into primitive endoderm-like cells (Robertson, 1987). First, we confirmed by immunostaining that undifferentiated F9 cells contain PECAM-1 (Fig. 7A); PECAM-1 is clearly found localized to the cell–cell sites of contact. F9 cells were then induced to differentiate with 1 μM retinoic acid over a 4-day period from which we isolated RNA every 24 h. Transcripts of markers of the undifferentiated cell type, such as Oct-4 and Sox-2, would be expected to decrease upon differentiation, and markers of differentiation, such as tPA, expected to increase (Behrendtsen *et al.*, 1995). RT-PCR analysis of this RNA clearly indicated PECAM-1 levels dropped to almost undetectable levels in the differentiated cells after 4 days of RA induction (Fig. 7B). Primitive endoderm-like cellular differentiation clearly occurred as tPA transcripts appeared and Sox-2 transcripts disappeared over the 4 days of differentiation (Fig. 7B). Oct-4 transcript levels decreased gradually, probably reflecting the quantitative nature of this transcription factor in directing self-renewal or differentiation (Niwa *et al.*, 2000).

At the 5.5 dpc time point in development, the mouse embryo is well implanted in the uterine wall and is surrounded by a large mass of decidual tissue. The epiblast cells are no longer rounded and pluripotent, but are columnar in shape. We determined whether PECAM-1 was detectable by immunostaining paraffin-embedded sections. The embryo at this stage was completely negative for PECAM-1 (Fig. 8C), although it was found in certain cells of the decidual mass (Fig. 8B). The decidual mass staining, most likely the result of endothelial cells from maternally derived capillaries, was useful as a positive control. These results indicate that PECAM-1 expression disappears from the cells of the epiblast in their transition from their rounded morphology in the 4.5-day embryo to their columnar morphology in the 5.5-day egg cylinder.

DISCUSSION

In this report, we have characterized the expression of the first cell adhesion molecule—PECAM-1—that is specific to the ICM/epiblast of the mouse blastocyst. PECAM-1 expression is restricted to cell–cell borders of the ICM at the first visible signs of blastocoel formation, is subsequently confined to the epiblast at peri-implantation, and is then undetectable in the egg cylinder.

PECAM-1 was previously thought to be vascular-specific, first appearing in newly forming endothelial cells of the yolk sac at embryonic day 7.25, approximately 2.75 days after implantation (Baldwin *et al.*, 1994; Drake and Fleming, 2000). The first evidence that PECAM-1 expression

might occur much earlier in development, prior to endothelial cell differentiation, derives from previous work of ours incorporating the −15,000- to +60-bp region (relative to the transcription start site) of the mouse PECAM-1 gene driving the expression of Cre recombinase (Terry *et al.,* 1997). When such a transgenic mouse was crossed with a mouse homologous for a conditional VCAM allele, the progeny effectively resulted in a nonconditional VCAM knockout, suggesting that this transcriptionally active region of the PECAM-1 gene drives expression of Cre in progenitor cells of the embryo proper. In addition, another report recently identified PECAM-1 mRNA and protein in ES cells (Vittet *et al.,* 1996). While our studies were in progress, a report, primarily focused on the differentiation of ES cells into endothelial cells, briefly noted PECAM-1 expression in the blastocyst without further defining cellular localization or kinetics of expression (Redick and Bautch, 1999).

Numerous cell types of the vasculature express PECAM-1: monocytes, neutrophils, platelets, particular B-cell and T-cell subsets, and endothelial cells. Although a precise function for PECAM-1 in these cells is still unknown, it has potential roles in the inflammatory response (Muller *et al.,* 1993; Wakelin *et al.,* 1996), angiogenesis (DeLisser *et al.,* 1997b; Matsumura *et al.,* 1997; Yang *et al.,* 1999), vasculogenesis (Baldwin *et al.,* 1994), and some T-cell responses (Torimoto *et al.,* 1992; Zehnder *et al.,* 1995). PECAM-1-deficient mice are viable but exhibit an abnormal migration of leukocytes across vascular basement membranes (Duncan *et al.,* 1999) and display a prolongation in bleeding time (Mahooti *et al.,* 2000). The work reported here indicates that PECAM-1's function is not restricted to the vasculature, as the blastocyst develops a full 4 days prior to any vascular formation. However, as evidenced from the null mice, this expression is not essential for embryo viability.

PECAM-1-mediated cell adhesion is predominantly the interaction of its extracellular domains interacting with one another on neighboring cell surfaces (Sun *et al.,* 2000; Wong *et al.,* 2000) (i.e., homophilic adhesion). This is thought to be due to a "diffusion trapping" mechanism (Sun *et al.,* 2000) whereby PECAM-1 diffuses within the plane of the plasma membrane until it comes in contact with a PECAM-1 molecule on a neighboring cell. In endothelial cells *in vivo,* this results in the majority of PECAM-1 molecules accumulating at the cell–cell junctions between neighboring endothelial cells, the luminal and basal endothelial cell surfaces having much fewer PECAM-1 molecules (Albelda *et al.,* 1991). Cell–cell contact in the ICM covers the entire surface of each cell and concomitantly PECAM-1 appears to be uniformly distributed across these cell surfaces (as evidenced from our serial confocal scans through the PECAM-1 immunostained ICMs), presumably in homophilic interactions with PECAM-1 on neighboring cells of the ICM.

PECAM-1 is also capable of trans-heterophilic interactions (DeLisser *et al.,* 1994; Yan *et al.,* 1995). PECAM-1 immunostaining was apparent at the cell–cell borders between the ICM and polar TE but not at cell–cell junctions between adjacent polar trophectoderm cells (see Fig. 2B). Although we cannot rule out the possibility that PECAM-1 is expressed in the cells of the polar TE and exclusively localized to the TE:ICM interface, the lack of PECAM-1 at cell–cell junctions between neighboring cells of the polar TE suggests that this is not the case. Therefore, PECAM-1 staining at ICM-polar TE junctions is likely the result of ICM-derived PECAM-1 molecules interacting with a heterophilic ligand on the polar TE.

Recent work has begun to focus on the function of the cytoplasmic domain of PECAM-1. Amino acid residues surrounding two tyrosines located in exons 13 and 14 conform to immunoreceptor tyrosine-based activation motifs (ITAMs) and immunoreceptor tyrosine-based inhibition motifs (ITIMs) (Newman, 1999; Pumphrey *et al.,* 1999). SHP-1, SHP-2, SHIP, β-catenin, γ-catenin, and phospholipase C-γ1 all are capable of association with the cytoplasmic domain of PECAM-1 (Ilan *et al.,* 1999, 2000; Jackson *et al.,* 1997; Pumphrey *et al.,* 1999). In the blastocyst PECAM-1 may function through at least some of these molecules as SHP-2, β-catenin, and γ-catenin are all present in the blastocyst (Ohsugi *et al.,* 1997).

Exons 12, 14, and 15 are subject to alternative splicing which likely adds further complexity to the signaling function of PECAM-1 (Baldwin *et al.,* 1994; Sheibani *et al.,* 1999). The significance of PECAM-1 alternative splicing in any tissue is not understood though isoforms lacking exon 14 appear to favor homophilic binding and isoforms with this exon favor heterophilic binding (Yan *et al.,* 1995). Differential association of signaling molecules to particular spliced isoforms of PECAM-1 have not been extensively studied. It appears, however, that exon 14 is required for SHP-2 binding (Wong *et al.,* 2000). There does not appear to be tissue specificity of individual isoforms as the majority are found in all tissues that normally express PECAM-1 (Sheibani *et al.,* 1999). The blastocyst is no exception to this as we identified seven of the eight potential isoforms. From our analysis the Δ15 and Δ14,15 isoforms appear to be the most abundant in the blastocyst. This abundent expression of Δ15 in the undifferentiated cells of the ICM is intriguing given that an apparent dedifferentiation has been observed when the Δ15 isoform is stably transfected into an epithelial cell line (Sheibani *et al.,* 2000).

PECAM-1 expression in the early embryo, first detected at the initial stages of ICM formation and disappearing in the epiblast by the early egg cylinder, closely corresponds to the pluripotency of these cells. Here we define pluripotency as the ability to colonize the blastocyst and to form ES cells. Cells of both the ICM and early epiblast are capable of these actions but during the transition from late blastocyst to early egg cylinder this ability is lost (Brook and Gardner, 1997; Gardner and Brook, 1997). This same transition is marked by a morphological change from a solid ball of rounded cells in the early epiblast into a columnar epithelium in the egg cylinder epiblast. Contact with the basement membrane is required for the survival of these columnar epithelial cells (Coucouvanis and Martin, 1995). Our

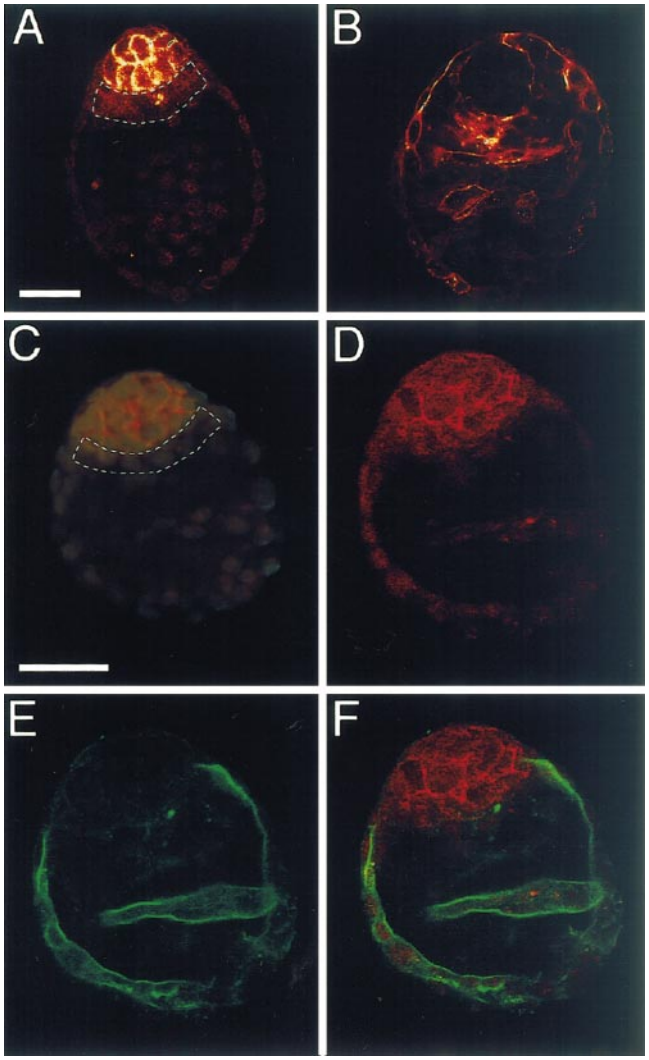


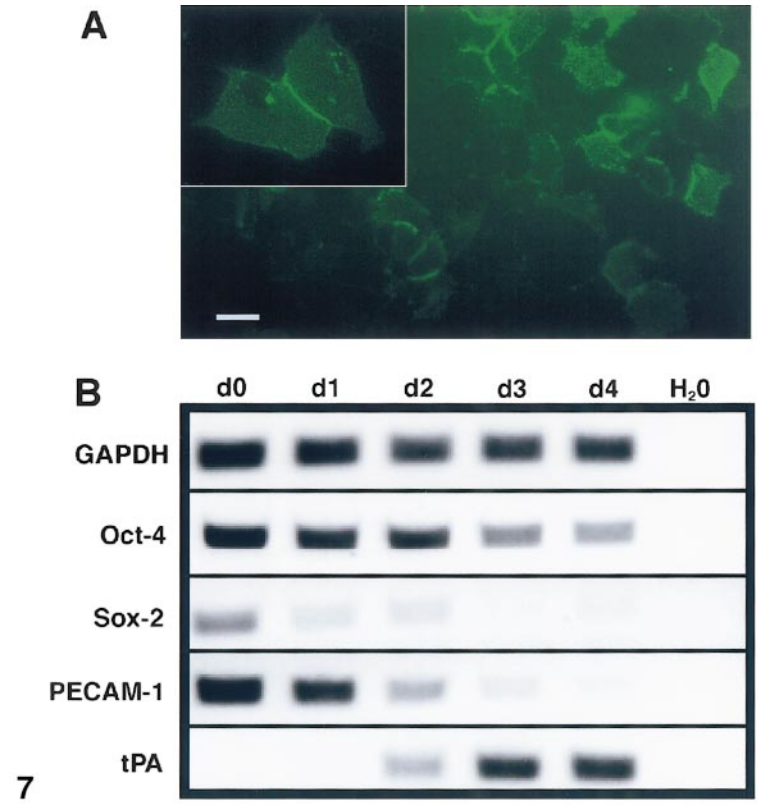
FIG. 6. Epiblast-specific staining of PECAM-1 in the 4.5-day embryo. All panels are confocal images of 4.5-dpc embryos. (A) An embryo immunostained for PECAM-1, (B) for type IV collagen, and (C–F), all from the same embryo, for PECAM-1 and type IV collagen. (C) The image from the Cy3 channel (PECAM-1) is overlaid with the DAPI signal for nuclear staining. (D) Solely the Cy3 channel, (E) the FITC (type IV collagen) channel, and (F) both Cy3 and FITC channels. The PECAM-1-negative region in the dashed area (A, C) represents the primitive endoderm. Images (A) and (B) are of equal magnification and images (C–F) are of equal magnification. The scale bars represent 25 μm.

work here distinctly shows that the PECAM-1-positive, rounded cells of the early epiblast lack any contact with the basement membrane (Fig. 6F) and once the columnar epithelial epiblast is formed PECAM-1 is no longer expressed (Fig. 8C). Furthermore, PECAM-1 expression disappears from F9 teratocarcinoma cells when these cells are induced to differentiate into basement membrane-synthesizing primitive endoderm-like cells (Fig. 7B). As basement membranes are well known to be a source of differentiation signals (Gumbiner, 1996), it is possible that PECAM-1's trans-homophilic interactions prevent attachment to the basement membrane and thereby interfere with potential differentiation signals.

Another possible role for PECAM-1 in maintaining pluripotency may involve its cytoplasmic domain, a region that apparently functions as an inhibitor and modulator of cell signaling (Ilan *et al.*, 1999; Newton-Nash and Newman, 1999). Self-renewal of ES cells involves the binding of LIF to the cell surface receptor gp130 and activation of STAT3 (Ernst *et al.*, 1999; Matsuda *et al.*, 1999; Niwa *et al.*, 1998). Interestingly, the elimination of SHP-2 binding to gp130 enhanced ES cell self-renewal (Burdon *et al.*, 1999). Thus PECAM-1, with its ability to bind SHP-2, may act to modulate SHP-2 binding to gp130.

PECAM-1 protein localizes to the cell–cell borders of the ICM at the onset of blastocyst formation (Fig. 3G). This, combined with the corresponding increase in PECAM-1 mRNA in the late morula/early blastocyst (Fig. 1) likely indicates an increase in the rate of transcription from the PECAM-1 gene, although at this juncture an increase in mRNA stability cannot be excluded. The apparent induction of transcription is independent of compaction, cytokinesis, and DNA synthesis, as inhibition of any one of these processes does not prevent PECAM-1 expression. This preimplantation expression pattern of PECAM-1 is likely coordinated by ICM-specific transcription factors. Oct-4 and Sox-2 are important regulators of ICM-specific transcription as both are involved in the ICM-specific expression of FGF4, UTF1, and osteopontin (Botquin *et al.*, 1998; Nishimoto *et al.*, 1999; Yuan *et al.*, 1995). However, there must be further complexity to this regulation as both Oct-4 and Sox-2 are expressed prior to blastocyst formation but the expression of FGF4, UTF1, and osteopontin, like that of PECAM-1, only begin upon blastocyst formation. For the PECAM-1 gene, there is indirect evidence from the PECAM-1/Cre mice (mentioned above and in Terry *et al.*, 1997) that the 15-kb 5′ flanking region is sufficient to drive ICM expression of PECAM-1. Sequencing of this region, which is 85% complete, has yet to identify any potential Sox-2 or Oct-4 binding sites. This opens up the intriguing possibility that ICM-specific PECAM-1 expression is controlled by an as yet undefined mechanism. PECAM-1 promoter studies in F9 teratocarcinoma cells, which are currently in progress, will allow us to identify the mechanisms involved in ICM-specific PECAM-1 transcription.

In summary, we have characterized the expression of PECAM-1 in the preimplantation embryo. PECAM-1 was previously thought to be restricted to cells of the vasculature, however, its ICM-specific expression now provides a nonvascular cell system to study its as yet unknown function. Furthermore, this is the first characterization of an ICM-specific cell adhesion molecule in the mouse blastocyst. Notwithstanding a potential role in maintaining pluripotency, PECAM-1's expression pattern makes it a



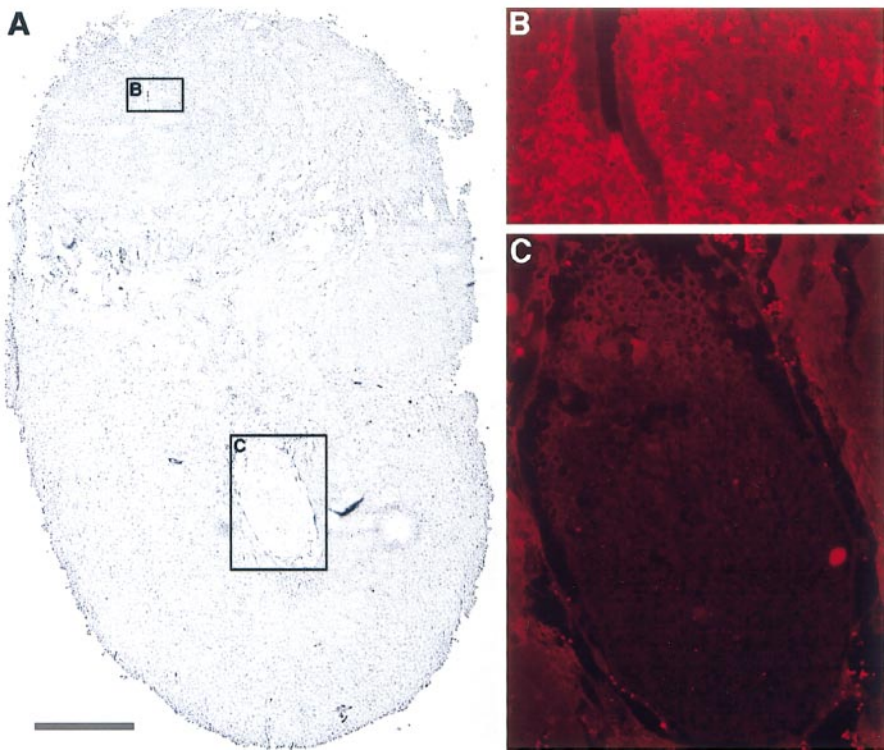


FIG. 7. PECAM-1 is found in undifferentiated but not in retinoic acid-induced differentiated F9 teratocarcinoma cells. (A) Undifferentiated F9 cells immunostained for PECAM-1. The scale bar represents 50 μm, the inset is a 2-fold magnification. (B) RT-PCR analysis of GAPDH, Oct-4, Sox-2, PECAM-1, and tPA mRNA levels are shown at 24-h intervals over the span of 4 days of 1 μM retinoic acid treatment.

FIG. 8. PECAM-1 expression is not detected in the egg cylinder stage embryo at embryonic day 5.5. (A) A DIC image of a 5.5-dpc embryo (enlarged in C) is shown embedded in the decidual mass. (B, C) Regions of (A) magnified 5-fold that have been immunostained with the PECAM-1 antibody, Mec 13.3. The scale bar represents 250 μm.

good marker of these cells, a practical aspect of which may be the use of this cell surface antigen in cell sorting for pluripotent cells. Future studies of PECAM-1 in the ICM and in ES cells should yield novel insights into the biology of these unique cells.

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